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Original Article

Gene expression of IQGAPs and Ras families in an experimental mouse model for hepatocellular carcinoma: a mechanistic study of cancer progression

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Abstract: IQGAPs genes play critical role in either induction or suppression of cancer and its progression, however the relationship between Ras genes and these genes are still unclear. In this study, we tried to understand the mechanistic action of IQGAPs genes and its correlation with Ras genes in mouse hepatic cancer model. The genetic expressions of IQGAP1, IQGAP2, IQGAP3, Hras, Kras, Nras, Mras, Caspase3, and BAX were followed in both hepatocellular carcinoma and normal liver cells of Balbc mice. Genotoxic agent diethylnitrosamine (DEN)-induced hepatic cancer model was induced in male mice and recorded the occurrence of hepatocellular carcinoma by morphological and histological changes in the liver. It was observed that mRNA expressions of IQGAP1, Hras, Kras, Nras, Mras, Caspase3, and BAX genes were highly elevated in hepatocellular carcinoma cells when compared with normal liver cells, additionally their expressions increased by concentrating the dose of DEN. While, the expressions of IQGAP2 and IQGAP3 were significantly decreased in hepatocellular carcinoma cells when compared with normal liver cells, as well as their expressions decreased more with increasing the dose of DEN. It was concluded from this study that IQGAP1 has a strong signaling relationship with Ras genes in induction of cancer and it is considered as a key gene for induction or suppression of the hepatocellular carcinoma.

Keywords: IQGAPs, IQGAP1, ras, DEN, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is responsible for between 500,000 and 1 million worldwide deaths annually. HCC etiologies are diverse and include chronic hepatitis B (HBV) and C (HCV), chronic excessive alcohol consumption, steatosis, diabetes, and exposure to toxic agents such as aflatoxin B1, or any hepatic disease associated with cirrhosis [1]. HCC develops when there is a mutation in the cellular machinery that causes the cell to divide at a higher rate, resulting in the cell to avoid apoptosis [2]. Additionally, a number of host factors such as male sex, older age at infection, long disease duration, excessive alcohol consumption, and high liver iron overload have been reported to influence disease progression. In particular, chronic infections of HBV and/or HCV can aid the development of HCC by repeatedly causing the body's own immune system to attack the liver cells [3]. HCV infection afflicts more than 170 million people worldwide, with the great majority (~85%) of patients developing chronic HCV infection [4].

N-nitrosodiethylamine (DEN) is often used as a carcinogenic reagent and the target organ in

which DEN induces malignant tumors is species-specific. Mice mainly develop not only liver cancer, but also gastrointestinal [5], skin, respiratory [6], and haematopoietic cancers [7]. The carcinogenic capacity of DEN is situated in its capability of alkylating DNA structures. DEN works in a dose-dependent manner [8]; a single low initiation dose not lead to the formation of neoplasms, while administration of a high dose induces HCC after a period of latency.

Intriguingly, the applications of gene expression patterns are well-established that they can accurately predict the clinical outcome of HCC at the time of diagnosis and therapy. Expression of 406 genes was highly correlated with length of survival with strong statistical significance. The outcome of hierarchical cluster analysis of the HCC with the 406 survival genes was similar to the previous analysis with all the genes [9].

Isoleucine-glutamine-motif containing GTPase-activating proteins (IQGAPs) is a small subgroup of evolutionally conserved superfamily of GTPase-activating proteins [10]. The IQGAP family is found in eukaryotes from yeast to mammals and it is named for their isoleucine-glutamine (IQ) and GTP-activating protein (GAP) domains. GAPs play a crucial role in the Ras superfamily regulation. Ras proteins act as signalling switchers by cycling between active GTP-bound and inactive GDP-bound states. GAPs negatively regulate Ras proteins by catalyzing the switch from the GTP-active to GDP-inactive state [1].

The IQGAP members contain multiple protein-protein interaction motifs [11]. Intriguingly, the IQGAPs regulate distinct cellular processes, including cell adhesion and migration as well as extracellular signals and cytokinesis. In human beings, there are three IQGAPs; IQGAP1, IQGAP2, and IQGAP3. IQGAP1 is the best characterized protein of this family and it is ubiquitously expressed, unlike IQGAP2 and IQGAP3, in hepatocytes [12]. In addition to four IQ domains, IQGAP1 contains a calponin homology domain, a polyproline interaction domain, a RasGAP-related domain (GRD), and a RasGAP-C carboxy-terminal sequence common to IQGAP family members [13, 14]. IQGAP1 assists in both ERK- and β-catenin-dependent signalling. IQGAP1 binds B-Raf, MEK and ERK to facilitate their sequential activation and propagation of the MAPK cascade [15, 16]. Constitutive activation of the MAPK pathway is a common oncogenic trigger in several cancers, notably those caused by Ras and B-Raf activating mutations. Whilst IQGAP1 possesses a Ras-GRD, it is not yet clear whether IQGAP1 binds to Ras. Early studies found no detectable binding with Ras family members H-Ras or R-Ras [17, 18], but positive interaction with active M-Ras. Furthermore, IQGAP3, not IQGAP1, was recently shown to interact with activated H-Ras and found to be an upstream regulator of ERK-dependent proliferative signaling [19]. Therefore, IQGAPs may bind to alternate Ras members and this selectivity may determine the fate of particular Ras-mediated signals.

This study aimed at evaluating the genetic expressions of IQGAP family (IQGAP1, IQGAP2, and IQGAP3), Ras family (Hras, Kras, Nras, and Mras), and apoptotic members (Caspase3 and BAX) in DEN-induced mice and normal controls to examine the mechanistic action of these genes in hepatocellular carcinoma progression.

Materials and methods

Animals

A total of eighty 21-day-old male Balbc mice were purchased from the Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia and housed in polycarbonate cages in an animal facility certified by the Association for Assessment and Accreditation of Laboratory Animal Care. Maintained in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, all of the mice were fed NIH-07 diet rodent chow and received water ad libitum. The animals were allowed to acclimate for a week prior to the experiments.

Chemicals

DEN was purchased from Sigma Chemical, St. Louis, MO, USA.

Experimental design

After acclimation, the 4-week-old mice were randomly divided into six experimental groups. In the 4-week treatment group, mice were treated with DEN dissolved in saline by i.p. injection at the dosages of 0, 50, and 75 mg/kg on

Table 1. Primer sequences of mouse genes for IQGAPs, Ras, Caspase3, and BAX

NM_001033484.1 Mus musculus IQ motif containing GTPase activating protein 3 (Iqgap3), mRNA	F: CCTCCGGAATGGAGTGCTAC
	R: CTGCAAGGTGACCAGAGTGT
NM_027711.1 Mus musculus IQ motif containing GTPase activating protein 2 (Iqgap2), mRNA	F: GGAAGGGGTTCAAACAACGC
	R: TGTCACCATTCGGAACCAGG
NM_016721.2 Mus musculus IQ motif containing GTPase activating protein 1 (Iqgap1), mRNA	F: TTCAAGGTCTCTGCGCTTCC
	R: GAGGCCGGTAGCCTTGTATC
NM_008624.3 Mus musculus muscle and microspikes RAS (Mras), mRNA	F: GGCGCTTTGTTCCGTTCC
	R: TATTGTTCCCGCATGGCACT
NM_021284.6 Mus musculus v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (Kras), mRNA	F: AGACACGAAACAGGCTCAGG
	R: GCATCGTCAACACCCTGTCT
NM_010937.2 Mus musculus neuroblastoma ras oncogene (Nras), mRNA	F: GGGGTCTCCAACAGCTTCTC
	R: AGGTCTCCTCCAGGGATGTC
NM_008284.2 Mus musculus Harvey rat sarcoma virus oncogene (Hras), transcript variant 1, mRNA	F: CTGTCCTGACACCAGGCTC
	R: ATGGACCCTCCTGTAGCCAT
NM_009810.3 Mus musculus caspase 3 (Casp3), transcript variant 2, mRNA	F: GGGGAGCTTGGAACGCTAAG
	R: CCGTACCAGAGCGAGATGAC
NM_007527.3 Mus musculus BCL2-associated X protein (Bax), mRNA	F: CTGGATCCAAGACCAGGGTG
	R: CCTTTCCCCTTCCCCCATTC

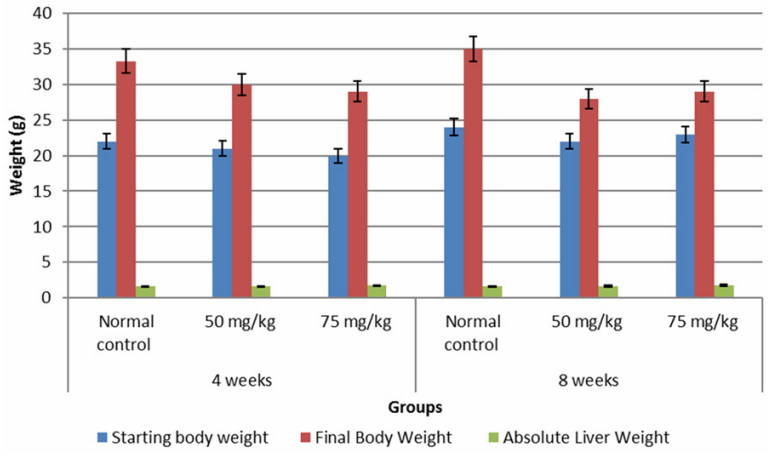

Figure 1. Body and liver weights in Balbc mice.

experimental days 0, 7, 14, and 21 (groups 1-3). In the 8-week treatment group, i.p. injections were given as above on experimental days 0, 50 and 75 mg/kg (groups 4-6). At experimental day 233, mice were killed by CO_2 asphyxiation and exsanguinations, weighed, and necropsied. The livers were removed *in toto*, weighed, and examined for the presence of grossly visible lesions. They were then separated by lobe and cut into 1- to 2-mm slices. One from each lobe was fixed in formalin for 48 to 72 hours and then embedded in paraffin to give a total of three paraffin blocks per animal. Serial sections from each block were stained with hematoxylin and eosin (H&E), hepatic preneoplastic and neoplastic lesions were classified according to the pathology of the mouse [20].

Quantitative analysis of altered hepatocellular foci

Quantitative analysis of AHF was accomplished using a two-dimensional evaluation. Whole H&E staining sections were scanned by ScanScope CS. Images were then viewed with Aperio's image viewer software (ImageScope), on which the quantitative analyses were performed. The number and volume of each foci (eosinophilic, clear, and basophilic) and total volume of liver sections were quantified. Because large hepatic tumors were recognized in one animals (one animal in the 75 mg/kg for 8 weeks group), these areas were not included in the total volume of liver sections of these animals. The multiplicity of AHF is reported as numbers per centimeter squared and the volume of foci as the percentage of total liver volume (%).

RNA extraction and cDNA synthesis

Total RNA from the liver tissue homogenate was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and quantified by measuring the absorbance at 260 nm. The RNA quality was determined by measuring the 260/280 ratio.

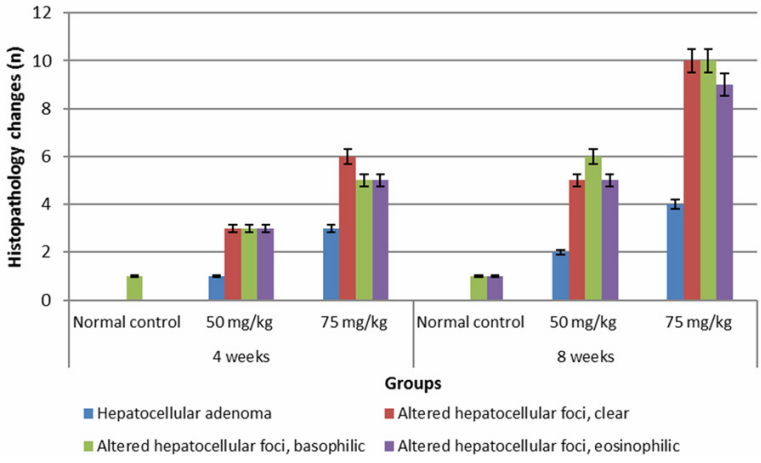

Figure 2. Incidence of histopathological changes of the liver in Balbc mice.

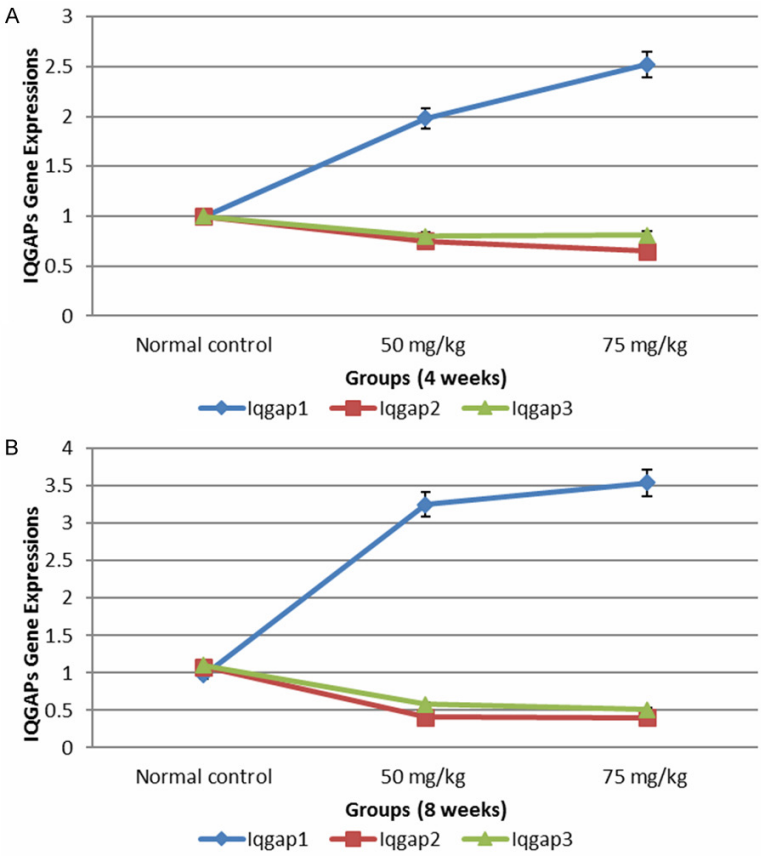

Figure 3. (A, B) The expression of IQGAP genes in liver tissues of different treated groups after 4 weeks (A) and 8 weeks (B).

Quantification of mRNA expression by real-time polymerase chain reaction

Quantitative analyses of mRNA expressions of the target genes which are considered as markers for liver fibrosis and injury were performed by RT-PCR through subjecting the resultant cDNA from the above preparation to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems). The 25 µl reaction mixture contained 0.1 µl of 10 µM forward primer and 0.1 µl of 10 µM reverse primer (40 µM final concentration of each primer), 12.5 µl of SYBR Green Universal Master mix, 11.05 µl of nuclease-free water, and 1.25 µl of cDNA sample. The primers used in the current study were chosen from pubmed.com (http://www.ncbi.nlm.nih.gov/tools/primer-blast) as listed in **Table 1**. Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. The real-time PCR data have been analyzed using the relative gene expression (i.e., ÄÄCT) method, as described in Applied Biosystems, User Bulletin No. 2. Briefly, the data are presented as the fold change in gene expression normalized to the endogenous reference gene (GAPDH) and relative to a calibrator.

Results

General observation, body and liver weight

The final body weight and absolute and relative liver weights are illustrated in **Figure 1**. During the experimental period, two mice in the 4 weeks 50 mg/kg treatment group (group 2), three mice in the 4 weeks 75 mg/kg treatment group (group 3), four mice in the 8 weeks 50 mg/kg treatment group (group 5) and four mice in the 8 weeks 75 mg/kg treatment group

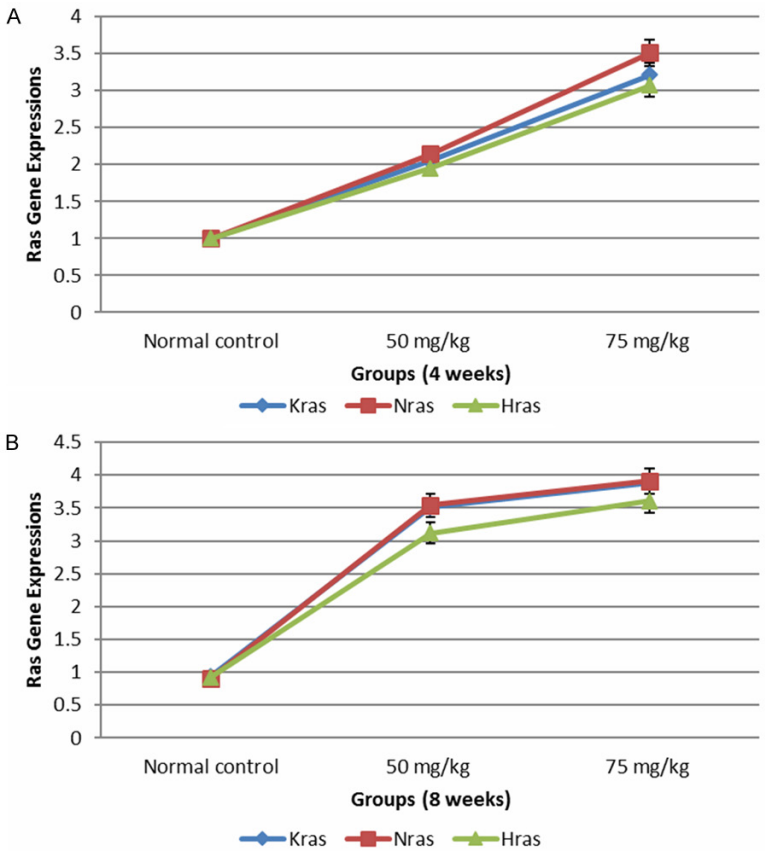

Figure 4. (A & B) The expression of Ras genes in liver tissues of different treated groups after 4 weeks (A) and 8 weeks (B).

(group 6) died. The causes of death could not be determined at necropsy. Final body weights were significantly decreased in the groups 3, 5, and 6 as compared with the control group. Measurement of organ weight revealed that the relative liver weight was significantly increased in groups 3 and 6 as compared with the control group.

Histopathological examination

Liver morphology of Balbc mice either normal or treated by DEN is illustrated in (**Figures 2**, **6**, **7**) summarizes the results of the histopathological examinations. Hepatocellular adenomas, holangiomas, and hemangiomas were observed in group 6. A hepatocellular adenoma was also identified in groups 3 and 5. In all DEN treatment groups, eosinophilic, clear, and basophilic AHF was identified. The incidences of clear and basophilic AHF increased in a dose-dependent manner and were statistically significant as compared with control values for groups 2, 3, 5 and 6. Eosinophilic AHF was also positively correlated to dose, but only those identified in group 6 were statistically significant. In addition, biliary cysts were identified in most of the group 6 animals.

In the 8-week treatment groups, the volume of total foci was significantly increased from the low-dose group compared with the control group, and basophilic AHF accounted for more than 50% of the total AHF. In the 4-week treatment groups, the volume of total foci was significantly higher in group 6, and more than half of the total AHF was basophilic.

Gene expression level

The genetic expressions of IQGAP1, raps (H, K, N and M), Caspase 3, and BAX were significantly higher in DEN-induced cancer group compared to normal control (NC) group asshownin(**Figures3**-**5**).Furthermore,theirexpressions increased by increasing the DEN dose. While, the expressions of IQGAP2 and IQGAP3 genes were significantly lower in all animal treated with DEN ($P<0.001$) compared to those of NC group and their expressions decreased by increasing the DEN dose as shown in **Figure 3**.

Discussion

The development of cellular and molecular biological techniques, many molecular markers related to invasion, metastasis, recurrence, and survival have been explored. A large number of molecular factors have been shown to associate with the invasiveness of HCC [21]. Another important aspect is the analysis adhesion molecules, proteinases as well as other molecules have been regarded as biomarkers for the HCC, and are related to prognosis and therapeutic outcomes [4]. The cellular changes that lead HCC, the etiologic agents responsible

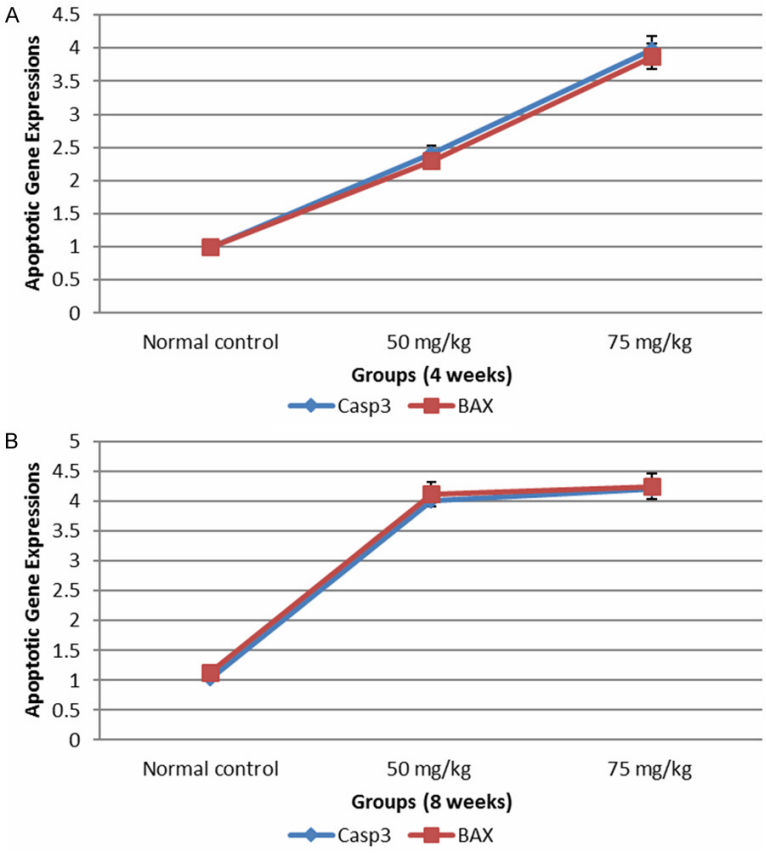

Figure 5. (A & B) The expression of Casp3 and BAX genes in liver tissues of different treated groups after 4 weeks (A) and 8 weeks (B).

for the majority of HCC and the molecular pathogenesis of HCC is not well understood [22]. Despite considerable progress in using clinical and pathologic diagnosis of HCC to predict patient survival and responses to therapy, a number of issues remain unresolved.

With few exceptions, cluster memberships of each tumor remained the same in the hierarchical cluster dendrograms, highlighting again the robustness of the predicted HCC subclasses and their association with the length of survival [9].

To disclose detailed genetic mechanisms in HCC with a view toward development of novel therapeutic targets, Wang et al. [23] make identification and validation of a novel gene associated with the recurrence of human HCC. Also, Hiroshi et al. [24] analyzed expression profiles of 20 primary HCCs and their corresponding noncancerous tissues by means of cDNA microarrays. Up-regulation of mitosis-promoting genes was observed in the majority of the tumors examined. Some genes showed expression patterns in hepatitis B virus-positive HCCs that were different from those in hepatitis C virus-positive HCCs; most of them encoded enzymes that metabolize carcinogens and/or anticancer agents [22]. Furthermore, they identified a number of genes associated with malignant histological type or invasive phenotype. Accumulation of such data will make it possible to define the nature of individual tumors, to provide clues for identifying new therapeutic targets, and ultimately to optimize treatment of each patient [24].

The mechanisms of tumorigenesis *in vivo* and *in vitro* are poorly understood. Overwhelming evidence indicates that specific mutated forms of the p21.sup.ras gene (ras) contribute to tumorigenesis [25]. When analyzed in a variety of neoplasms, the ras genes frequently contain characteristic point mutations that result in constitutive activation of p21.sup.ras. Expression of abnormally high levels of normal p21.sup.ras as a result of gene amplification or regulatory sequence mutations may also contribute to the transformation of normal cells to cancerous cells [26].

The ras genes which encode 21 kDa proteins are expressed ubiquitously and are found associated with the plasma membrane in the cytoplasm of the cell. p21.sup.ras is a guanine nucleotide-binding protein which catalyzes the hydrolysis of bound guanine triphosphate (GTP) to guanine diphosphate (GDP) and is believed to be a key component of a complex intracellular signal transduction pathway from the plasma membrane to the nucleus. It is active when bound to GTP and inactive in its GDP-bound state [25, 26].

The current study deduced that the genetic profile of IQGAP1, ras (H, K, N and M), caspase 3, and BAX were higher in DEN-induced cancer group compared to normal control (NC) group.

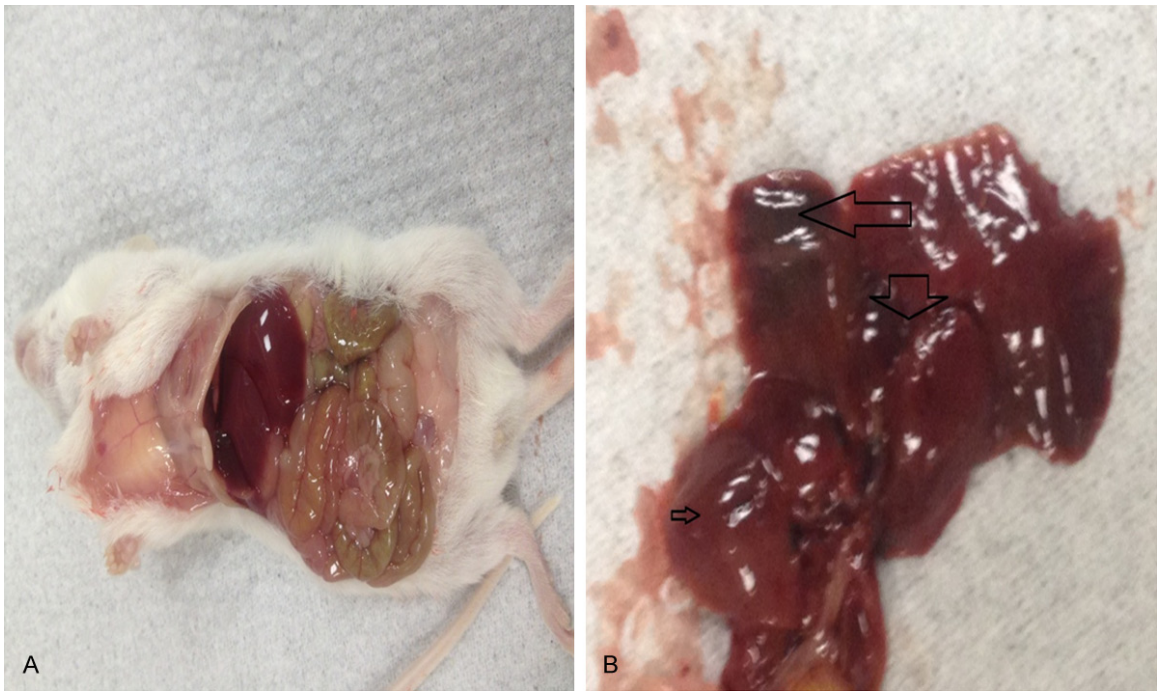

Figure 6. Morphology of Balbc mice liver treated by DEN (A) is normal control and (B) is treated by 75 mg/kg DEN 8 weeks.

While, the expressions of IQGAP2 and IQGAP3 genes were lower in all DEN-induced animal models compared to those of NC group. These observations agreed with previous studies indicated that the ras gene is the most frequently identified oncogene in human cancer, but oncogenic p21.sup.ras is resistant to the action of known mammalian ras-GAPs [27, 28].

There is mounting evidence to suggest a role for IQGAP1 in cancer progression. The many cellular processes that IQGAP1 orchestrates are highly relevant to cancer. IQGAP1 protein is not mutated but does display an unusually high expression in several cancer types, correlating with poor outcome [29].

Additionally, Colin et al. [21] have reviewed published data which implicate IQGAPs in cancer and tumorigenesis where the cumulative evidence suggests IQGAP1 is an oncogene while IQGAP2 may be a tumor suppressor. Colin et al. [21] discussed evidence derived from human neoplasms and mouse models of cancer, which more directly identify the involvement of IQGAP1 and IQGAP2 in neoplastic transformation and metastasis. The inverse correlation of IQGAP1expression with cancer progression suggests that IQGAP2 may be a tumor suppressor. This hypothesis was supported by studies which showed reduced expression of the IQGAP1 gene in hormone-refractory prostate cancer and. However, other reports contradict these findings as they observe overexpression of IQGAP1 in tissue from cancers of the colon and prostate [29]. Thus, while there is evidence to suggest that IQGAP2 acts as a tumor suppressor, more thorough investigations are required in order to verify this postulate and ascertain whether it pertains only to selected neoplasms.

In yet another aspect, the invention features a method of detecting a neoplastic cell in a tissue. The method involves measuring IQGAPs expression wherein decreased or increased expression of these genes product compared to a standard or known level of expression associated with normal tissue is indicative of the presence of a neoplastic cell. IQ-domain GTPase-activating proteins (IQGAPs) are an evolutionary conserved family of multi-domain proteins. These proteins regulate distinct cellular processes including cell adhesion, cell migration, extracellular signals and cytokines [3].

The name IQGAP1 is derived from two of its many interacting domains: four centrally located IQ motifs and a C-terminal Ras-GAP-related

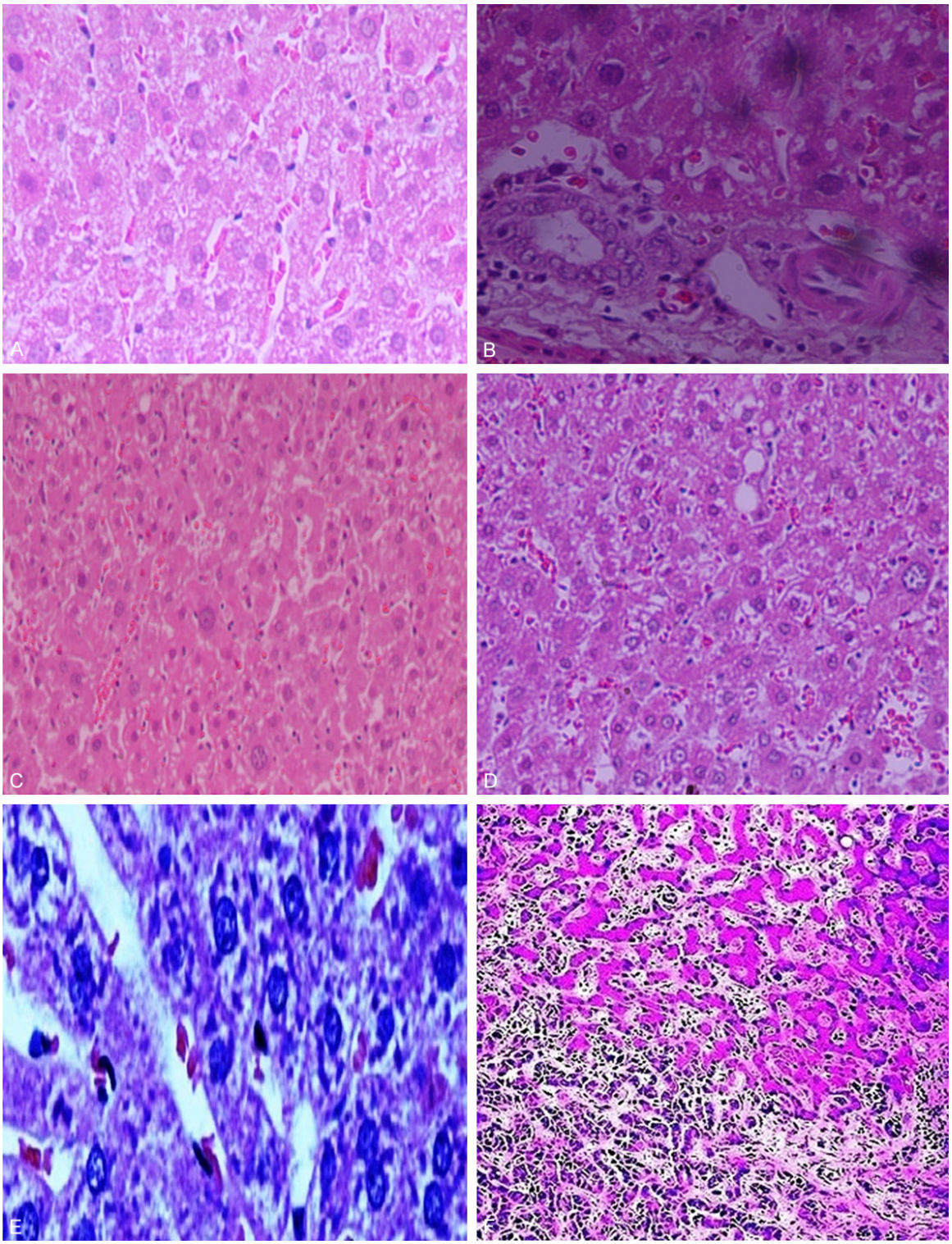

Figure 7. Formation of altered hepatocellular foci (AHF) and tumors in the liver of Balbc mice treated with diethylnitrosamine. (A-D) The lesions of a hepatocellular adenoma (C and D), a cholangioma (D), and a hemangioma (C-F). (H) Typical basophilic, clear, and eosinophilic AHF, along with a biliary cyst. (A and B) Hematoxylin and eosin stained.

domain. Traditional GTPase-activating proteins (GAPs) are regulatory effectors of GTPases such as those of the Ras superfamily. Ras proteins act as signalling switches by cycling

between active GTP-bound and inactive GDP-bound states. GAPs negatively regulate Ras proteins by catalyzing the switch from the GTP-active to GDP-inactive state [21].

IQGAP1, however, does not act as a traditional GAP and is able to stabilize small Rho GTPases Rac1 and Cdc42 in the GTP-bound state [19]. IQGAP1 activity itself is controlled by the ubiquitous calcium-binding protein, calmodulin, via the IQ motifs-classical calmodulin-interacting domains found in numerous proteins. It was hypothesized that calmodulin alters IQGAP1 activity by inducing a conformational change which influences IQGAP1-protein interactions and/or the subcellular localization of IQGAP1 [30]. IQGAP1 also possesses a calponin-homology domain (CHD) that binds F-actin, putative coil-coil homodimerisation domains, a tryptophan repeat motif (WW) of undefined function and a RasGAP_C-terminus (RGCt) that binds numerous proteins including APC, E-cadherin and β-catenin. The broad range of interacting partners places IQGAP1 as a key mediator of numerous cellular processes and signalling pathways [21].

IQGAP1 is a member of a three-isoform family of proteins that also includes IQGAP2 [31] and IQGAP3 [23]. IQGAP1, the most studied, binds calmodulin, cross-links actin filaments, integrates signaling networks [12], and regulates cell-cell contacts and the capture of microtubule plus-ends via association with CLIP-170 [30].

Eric et al. [32] presented a novel role for IQGAP1 in the regulation of exocytosis, where they investigated whether mammalian IQGAP1 associates with the exocyst-septin complex and influences secretion in a CDC42-regulated fashion. They found that overexpression or activation of CDC42 by mastoparan; dominant-active mutations or IQGAP1 expression disrupted the endogenous association of IQGAP1 with the exocyst-septin complex and blocked secretion. This effect appeared to be mediated by the C-terminus of IQGAP1, which binds CDC42 and inhibits secretion [21].

By contrast, the N-terminus of IQGAP1 binds to the exocyst-septin complex, enhances secretion and abrogates the inhibition caused by CDC42 or the depletion of IQGAP1, perhaps via the involvement of the N-terminus in protein synthesis, as indicated by pulse-chase experiments. These data raised a possibility that IQGAP1 serves as a regulator of secretion by acting as a conformational switch [32]. The level of expression of IQGAP genes and mRNAs are frequently altered in neoplasia. IQGAP1 has been proposed to be an oncogene. Consistent with this postulate, comparison of the genetic profiles of tumors with those of normal tissue, and comparison of more aggressive cancers with less aggressive neoplasms, reveals that the IQGAP1 gene and/or mRNA are overexpressed in all analyses reported. Increased expression of IQGAP1 has been observed in several human neoplasms, including lung, colorectal cancers and oligodendroglioma. Analogous observations have been reported in mouse models [21]. IQGAP1 gene was amplified in HSC39 and HSC40A gastric cancer cell lines. This amplification corresponded to an increase in both IQGAP1 mRNA and protein, compared with normal gastric cell lines, and an accumulation of IQGAP1 protein at the cell membrane [3].

IQGAP2 had also generated several “hits” in genetic screens comparing normal with neoplastic tissue. The results, however, were less unequivocal than those for IQGAP1. IQGAP2 expression was lost from 5/9 gastric cancer cell lines due to aberrant methylation of the IQGAP2 promoter [3].

Constitutive activation of the MAPK pathway is a common oncogenic trigger in several cancers, notably those caused by Ras and B-Raf activating mutations. Whilst IQGAP1 possesses a Ras-GRD, it is not yet clear whether IQGAP1 binds to Ras. Early studies found no detectable binding with Ras family members H-Ras or R-Ras [17] but positive interaction with active M-Ras [18]. Furthermore, IQGAP3, not IQGAP1, was recently shown to interact with activated H-Ras and found to be an upstream regulator of ERK-dependent proliferative signaling [19]. Therefore, IQGAPs may bind to alternate Ras members and this selectivity may determine the fate of particular Ras-mediated signals.

Finally, it was concluded that IQGAP1 has a strong signaling network with Ras (H, K, N and M) genes in induction of cancer. In addition, IQGAP/Ras signaling pathway may be considered as a key for induction or suppression of the HCC.

Acknowledgements

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Disclosure of conflict of interest

None.

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